EFFECT OF TIME AND TEMPERATURE ON BUTANOL PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) BY ANAEROBIC FERMENTATION USING *CLOSTRIDIUM BEIJERINCKII*

SITI HAZRIAH BINTI HAMZAH

UNIVERSITI MALAYSIA PAHANG

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ABSTRACT

Owing to the increasing volume of palm oil mill effluent (POME) wastewater generated, disposal remains as perennial problem and its bioconversion has been considered as an option for pollution control (C.N. Hipolito *et al.,* 2008). Thus, the availability of an inexpensive raw material; POME which will used in this research is essential if solvent fermentation is to become economically viable. In Malaysia, POME represents an alternative cheap carbon source for fermentation processes that is attractive in both economic and geographical considerations. The mainly research purpose is to study the production of butanol from the anareobic fermentation of solventogenic bacteria (*clostridium beijerinkii,* ATCC 51743) by using POME as the fermentation media. The scope of the research is to study the effect of fermentation time and temperature to the butanol production. The fermentation is based on batch fermentation in schott bottle. It is an anaerobic fermentation and the strain, *Clostridium Beijerinckii* (ATCC 51743) was treated strictly in an anaerobic condition. Optimum conditions that were maintained in this research are the substrate concentration and agitation rate. POME and Reinforced Clostridia Media (RCM) were used as the growth medium in this batch culture. The fermentation temperature that were used in this study are 35°C, 40°C and 45°C while the fermentation time that were used are 48 to 72 hours respectively. The results indicated that the concentration of butanol will decreases as the temperature increases. The highest concentration of butanol that produced by POME was 0.224 g L^{-1} at 35°C by 72 hours of fermentation. From this study, it is showed that POME can produce butanol at optimum fermentation temperature at 35°C in 72 hours of fermentation time. Hence, the result also showed that POME is viable to use as growth medium to produce butanol.

ABSTARK

Penambahan sisa buangan kelapa sawit (POME) yang terhasil, masalah pembuangan berterusan dan penukaran biologi sisa ini dianggap sebagai salah satu bentuk pencemaran (C.N. Hipolito *et al.,* 2008). Namun begitu, bahan asas yang murah sedia ada; POME yang diguna di dalam kajian ini sesuai untuk penapaian pelarut menjadi salah satu kajian yang ekonomi. Di Malaysia, POME merupakan alternatif yang murah sebagai sumber karbon untuk proses penapaian yang juga sesuai dalam segi ekonomi dan geografi. Tujuan utama kajian ini ialah untuk menkaji penghasilan butanol daripada proses penapaian tanpa oksigen menggunakan bacteria yang boleh menghasilkan pelarut (*clostridium beijerinkii,* ATCC 51743) dengan mengaplikasikan penggunaan POME sebagai media untuk penapaian. Skop kajian ini adalah kesan masa dan suhu penapaian terhadap penghasilan butanol. Penapaian ini adalah penapaian berbentuk terkumpul di dalam botol schott. Penapaian ini juga adalah penapaian tanpa oksigen dan bakteria yang digunakan adalah *Clostridium Beijerinckii* (ATCC 51743) yang juga bakteria yang hanya boleh hidup di dalam keadaan tanpa oksigen. Keadaan yang optimum dikekalkan di dalam kajian ini adalah kepekatan cecair dan tahap gerakan. POME dan Reinforce Clostridia Media (RCM) digunakan sebagai media tumbesaran di dalam pembentukan terkumpul ini. Suhu penapaian yang digunakan di dalam kajian ini adalah 35°C, 40°C dan 45°C sementara masa penapaian yang digunakan adalah 48 hingga 72 jam. Data menunjukan, kepekatan butanol akan menurun jika suhu meningkat. Kepekatan butanol yang paling tinggi yang dihasilkan daripada POME adalah 0.224 g L^{-1} pada suhu 35°C selama 72 jam penapaian. Kajian ini menunjukan, POME boleh menghasilkan butanol pada suhu penapaian optimum iaitu 35°C dalam masa 72 jam penapaian. Namun begitu, data kajian juga membuktikan POME boleh digunakan sebagai media tumbesaran untuk menghasilkan butanol.

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LIST OF ABBREVIATIONS

ABE - Acetone-Butanol-Ethanol

CPO - Crude Palm Oil

DNS - Dinitrosalicylic Acid

FFB - Fresh Fruit Bunch

FID - Flame Ionization Detector

GC - Gas Chromatography

HPLC - High Performance Liquid Chromatography

min - Minute

OD - Optical Density

POME - Palm Oil Mill Effluent

RCM - Reinforce Clostridia Medium

UV-VIS - Ultraviolet–visible Spectroscopy

LIST OF SYMBOLS

$ - dolar

% - percentage

cm - centimeter

ft - feet

g - gram

h - hour

kg - kilogram

L - liter

mL - mililiter

mm - millimeter

nm - nanometer

^{o}C - degree Celcius

rpm - revolutions per minute

w/v - weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Research Background

In the new era of the world, scientist and engineer were pushed by the world government to develop a technology to substitute the usage of fuel in transportable because of the crude for fuel processing is almost depleted. Also with the urgency of environmentalist, a green technology as an alternative of the usage of fuel is also being researching for almost decade.

Palm oil mill effluent (POME) treatment was being experimented in many ways to become treated or to become a product. The high compositions and concentrations of carbohydrate, protein, nitrogenous compounds, lipids and minerals in POME (Hwang *et al*., 1978; Phang, 1990; Habib *et al*., 1997) render it possible to reuse the effluent for biotechnological means. Production of butanol is one of the treatments that can be done to the POME; the alternatives fuel that being develop for the mean of green technology.

Various attempts were being made to achieve high yield of butanol production from POME using solvent fermentation seems impossible. Due to this fact, this paper is

being study to come up with the best parameter with the use of clostridium beijerinckii to yield higher percentage of butanol from POME using solvent fermentation technique.

1.2 Problem Statement

There has been an increased interest in research on the bioconversion of agricultural biomass into fuels and chemical feedstock's for two primary reasons, one being the limited supply of fossil fuels and petroleum, and the other, the increasing and fluctuating prices of oil. In order to overcome these problems, research is focused on developing bioconversion processes for fuels and chemicals (N. Qureshi *et al.,* 2008).

Owing to the increasing volume of palm oil mill effluent (POME) wastewater generated, disposal remains a perennial problem and its bioconversion has been considered as an option for pollution control (C.N. Hipolito *et al.,* 2008). Thus, the availability of an inexpensive raw material; POME which will used in this research is essential if solvent fermentation is to become economically viable. In Malaysia, POME represents an alternative cheap carbon source for fermentation processes that is attractive in both economic and geographical considerations.

Unfortunately based on researches in the market, the problem with the technology is to produce high yield of butanol from POME. Therefore, this study is focusing on finding the suitable parameters for solvent fermentation that can produce higher yield of butanol from POME using *clostridium beijerinkii*.

1.3 Objective

The objective of this research is to study effect of time and temperature on butanol production from palm oil mill effluent (POME) by anaerobic fermentation using *Clostridium Beijerinckii*.

1.4 Scopes

To achieve the objective, four scopes have been identified in this research:

i. To determine the growth pattern of the *Clostridium Beijerinckii* strain in POME substrate
ii. To study the effect of temperature (35 - 45°C) to the butanol production
iii. To study the effect of fermentation time (48 hours – 72 hours) to the butanol production
iv. To study the effect of the parameters on glucose consumption of the substrate

1.5 Rationale & Significance

The significant of this research is to study the possibility of POME as a good substrate for solvents fermentation and hence give an alternative substrate for the solvents fermentation purpose. Besides that, this study will help to establish a suitable parameter to overcome the lower yield of butanol production in solvent fermentation from POME.

CHAPTER 2

LITERATURE REVIEW

2.1 Palm Oil Mill Effluent (POME)

The production of palm oil results in the generation of huge quantities of highly polluting wastewater termed as Palm Oil Mill Effluent (POME).

The extraction process for crude palm oil (CPO) starts from the local palm oil mills throughout Malaysia. The mills processes FFB received from the oil palm plantations into CPO and other by-products. A schematic process flow of palm oil milling for the extraction of crude palm oil and sources of waste generation is shown in **Fig. 2.1**. Palm oil mills typically generate large quantities of extremely oily organic contented liquid (Industrial Processes and The Environment, 1999).

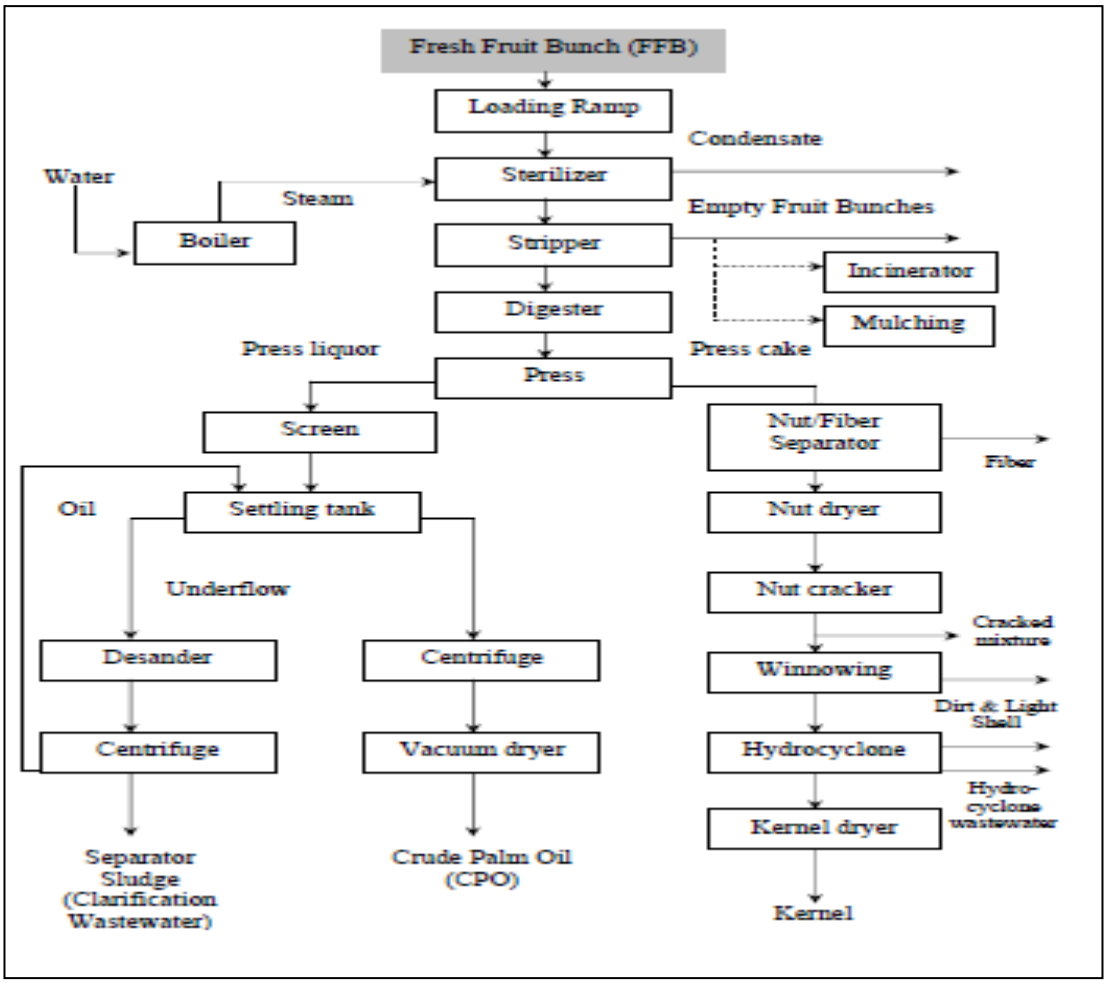

Figure 2.1 Process flow of typical palm oil milling (Industrial Processes and The Environment, 1999)

Large quantities of water are used during the crude oil extraction process. Up to about 1.5 cubic meters of water are characteristically used to process one tonne of FFB. From this quantity, about 50% of the water results in the POME, the other 50% being lost as steam, mainly through sterilizer exhaust, piping leakages, as well as wash waters (Oil Palm & The Environment A Malaysian Perspective, 1999). POME comprises a combination of the wastewaters which are principally generated and discharged from the following major processing operations as seen early in Fig. 2.1, such as; sterilization of FFB - sterilizer condensate is about 36% of total POME, clarification of the extracted CPO - clarification wastewater is about 60% of total POME and hydrocyclone

separation of cracked mixture of kernel and shell-hydrocyclone wastewater is about 4% of total POME.

Based on previous research by bachelor student, Einayah (2009), sugar compositions in POME are being analyzed by implemented high performance liquid chromatography (HPLC) function using capillary column Supelcosil LC-NH_2. **Table 2.1** and **Figure 2.2** showed the concentration of each sugar groups in POME.

Table 2.1 Sugar concentration in POME

Sample	Sugar Group	Concentration(g/L)
POME	Fructose	1.967
	Glucose	3.668
	Galactose	8.859
	Sucrose	2.24
	Lactose	1.313

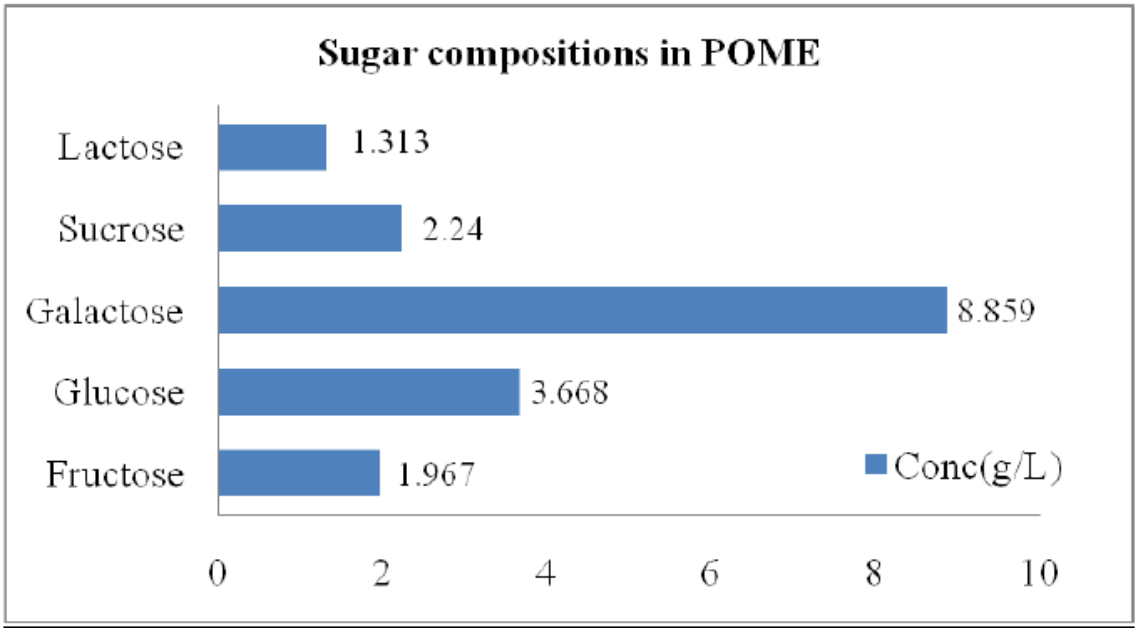

Figure 2.2 Sugar concentrations in POME

The possibility of reusing POME as fermentation media is largely due to the fact that POME contains high concentrations of carbohydrate, protein, nitrogenous compounds, lipids and minerals (Hwang *et al*., 1978; Phang *et al*., 1990; Habib *et al*., 1997; Suwandi *et al*., 1991; Wu *et al*., 2006b) pointed out the possibility of recovering and concentrating the available bioresources in POME by an ultrafiltration process in order for the concentrated bioresources to be reused more effectively as fermentation media. According to Wu *et al*. (2006b) POME and its derivatives have been exploited as fermentation media to produce various products/metabolites such as antibiotics, bioinsecticides, solvents, polyhydroxyalkanoates, organic acids as well as enzymes to varying degrees of success. The hydrogen production from POME during anaerobic treatment has also been intensively studied (Atif *et al*., 2005; Vijayaraghavan *et al*., 2006) since the generated hydrogen and its combustion products do not count as green house gases (Koroneos *et al*., 2004). However, it has been reported that POME also contains certain powerful water-soluble antioxidants, phenolic acids and flavonoids (Wattanapenpaiboon *et al*., 2003) that may inhibit the growth development in microorganisms (Lin *et al*., 2005; Uzel *et al*., 2005).

2.2 Butanol

Butanol is a 4-carbon alcohol originally central to a number of industrial chemical processes. It is now recognised as an important transport fuel - with superior characteristics to ethanol.

With four carbons, butanol has more energy than ethanol - 25% more energy per unit volume. Butanol has a lower vapour pressure and higher flashpoint than ethanol, making it easier to store and safer to handle. Butanol is not hygroscopic while ethanol attracts water. Ethanol has to be blended with petrol shortly before use. Butanol can be blended at a refinery without requiring modifications in blending facilities, storage tanks or retail station pumps. Butanol can run in unmodified engines at any blend with petrol.

Ethanol can only be blended up to 85% and requires engine modification. Unlike ethanol, butanol may also be blended with diesel and biodiesel. Butanol is less corrosive than ethanol and can be transported using existing infrastructures.

While current utilization strategies for biomass have focused on ethanol production, producing butanol instead of ethanol offers several advantages for biofuel-gasoline blending. With lower vapour pressure but higher energy content makes butanol safer for blending with gasoline as well as offering better fuel economy than ethanol-gasoline blends. In addition, with the higher tolerance to water contamination in gasoline blends and therefore butanol-gasoline blends are less susceptible to separation and that facilitates its use in existing gasoline supply and distribution channels. Therefore, optimizing ABE fermentation to enhance butanol production over ethanol appears to be the more commercially and technologically attractive option (C.N. Hipolito *et al.,* 2008).

2.3 Fermentation

2.3.1 Anaerobic Fermentation

Anaerobic fermentation is the process of fermentation without using any oxygen. One of advantages of the anaerobic process is the recovery of the useful matters such as solvents (Hwang *et al.,* 2004).

The most important economic factor in solvent fermentation is the cost of substrate, which made up about 60% of the overall cost of production. (Liew *et al.*, 2006). Biobutanol production is an anaerobic two-stage fermentation process where acetic and butyric acids, carbon dioxide and hydrogen are first produced in the acidogenic phase. Then the culture undergoes metabolic shift to solventogenic phase and acids are converted into acetone, ethanol and butanol. At the end of the fermentation, products are recovered from the cell mass, other suspended solids, and by-products (Pakkila *et al*., 2009).

2.3.2 Solvent Fermentation

Acetone-butanol-ethanol (ABE) fermentation by microbial is one of the oldest known industrial fermentations. It was ranked second only to ethanol fermentation by yeast in its scale of production, and is one of the largest biotechnological processes ever known. The actual fermentation, however, has been quite complicated and difficult to control. ABE fermentation has declined continuously since the 1950s, and almost all butanol is now produced via petrochemical routes. Butanol is an important industrial solvent and potentially a better fuel extender than ethanol. Current butanol prices as a chemical are at $3.75 per gallon, with a worldwide market of 370 million gallons per year. The market demand is expected to increase dramatically if green butanol can be produced economically from low cost biomass.

2.4 Solventogenic Clostridia

C. beijerinckii is a saccharolytic, strictly anaerobic, mesophylic, motile, rod-shaped bacteria with oval, sub-terminal spores. It exhibits peritrichous flagella. During fermentation, *C. beijerinckii* produces a number of products including acetate, butyrate, lactate, hydrogen gas, carbon dioxide, acetone, butanol, ethanol, acetoin and acetyl methyl carbonil. The morphology of the cell changes over the growth cycle of the organism; at early exponential phase, the cells are long, filamentous and very motile. As the culture approaches the solventogenic stage, which corresponds with the stationary phase, cells shorten, become plumper and exhibit a lower level of motility *C. beijerinckii* species are ubiquitous in nature and routinely isolated from soil samples (US Department of Energy Joint Genome).

C. beijerinckii has great biotechnological potential for the production of butanol, acetone, and/or isopropanol because of its broad substrate range (pentoses, hexoses, starch, and others), its sustained production of solvents well into log-phase, its stability with respects to strain degeneration and the adaptability it shows to continuous

processes. Pilot plant studies confirmed that *C. beijerinckii* grows well and is easy to handle in simple, inexpensive media that is realistic for industrial use. *C. beijerinckii* has also shown responsiveness for genetic improvement. The exceptional solvent productivity of the strain *C. beijerinckii*, produced after only one episode of mutagenesis, has demonstrated the enormous potential of derivatives of C. beijerinckii in solvent production (US Department of Energy Joint Genome).

The sequence of *C. beijerinckii* will make possible the application of DNA microarrays for gene expression profiling and comparative genomics in order to understand the phenotypic differences apparent between C. beijerinckii and other important saccharolytic strains, such as C. acetobutylicum. That may lead to the unraveling of the general principles of saccharide utilization and solvent production and therefore, to rational approaches to strain construction and optimization of the acetone-butanol fermentation.

CHAPTER 3

METHODOLOGY

3.1 Material

3.1.1 Bacterial strains and culture maintenance

Clostridium beijerinckii was used for these studies. Laboratory stocks of *C. beijerinckii* were maintained as spore suspensions in glycerol stock vial at -80°C. For prepare for pre-culturing the strain, spores were allowed to liquefy for 10 minutes at room temperature in anaerobic chamber to maintain the anaerobic condition. 1 mL of the stock culture was transferred into 150 mL Reinforced Clostridia Media (RCM) and incubated for 24 hours until it active to be used.

Streaking technique used to isolate pure *C. beijerinckii* strain from the active culture to be grown in Reinforced Clostridia Agar plate that has been sterilized. After streak the active strain on the sterilized agar plate, the plates were incubated for another 18 – 24 hours at 35°C in anaerobic condition. From this technique, the growth of *C. beijerinckii* can be explored, identified and studied.

3.1.2 Preparation of medium

RCM was used as pre-culture medium, as main culture and as control medium for batch fermentation. 38 g RCM powder was suspended in 1 liter of distilled water and brings to the boil to dissolve completely. The solution was sterilising by autoclaving at 121°C for 15 minutes.

While RCM used as control medium, Palm Oil Mill Effluent (POME) was used as experimental medium for the batch fermentation. POME was collected from Kilang Kelapa Sawit Felda Lepar Hilir, Gambang, Pahang. Fresh POME was sediment passively in heat resistant bottle and stored at 4°C for 24 hours. After 24 hours, the upper layer (supernatant) was decanted and lower layer (POME sludge) use as the experimental medium. The pH of POME is adjusted to 5.8 using 5M of NaOH and then was sterilized in autoclave at 121°C for 15 minutes.

3.1.3 Inoculum development

For producing pure culture of C. beijerinckii strain, after distinguish visible colonies of C. beijerinckii on the incubated agar plate, few loops of the colonies transferred to 150 mL of RCM in a flask and place in anaerobic condition for 18 – 24 hours at 36 ± 1 °C for inoculums development. After the incubation time, the culture broth was prepared for fermentation process. The culture broth was centrifuge for 10 minutes at 10000 rpm in microcentrifuge. The supernatant was decanted and re-suspend the cell with 100 ml of sterile saline solution, 0.85% (w/v) NaCl for cell washing. The cell washing was performed twice to wash out all the remaining broth from the cell. For the final cell suspension, the optical density (OD) value of 1.3 at 600nm was set and read the OD value using UV-VIS. If the OD value exceeds 1.3, add more saline solution. If the OD value less than 1.3, add more cell. Use the final cell suspension with OD value of 1.3 ± 0.1 as the inoculum for subsequent works (10% of the working solution).

3.2 Experimental Procedure

3.2.1 Batch fermentation

Batch fermentations were carried out in 500 mL screw-capped (300 mL of medium) and was been operate in hybrid incubator shaker. The medium contained RCM and POME at 243 mL adding up with 27 mL of sterilized distilled water for 90% concentration of substrate, and was autoclaved for sterilization condition. This was followed by the addition of final cell suspension at inoculation rate of 10%. After inoculation, the broth was spurge with filtered oxygen-free nitrogen gas for 30 minutes to maintain strict anaerobic conditions. The fermentation were experiment on two parameters on two different fermentation medium; fermentation time and fermentation temperature (**Table 3.1**) on optimum agitation rate (200 rpm) and concentration of substrate (90%). Samples were periodically withdrawn every 6 hours.

Table 3.1 Batch fermentation

		FERMENTATION	
RUNS	MEDIUM	TEMPERATURE, °C	TIME, hours
1	RCM	35	48
	POME		
2	RCM		72
	POME		
3	RCM	40	48
	POME		
4	RCM		72
	POME		
5	RCM	45	48
	POME		
6	RCM		72
	POME		